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NOTE TO THE EDITOR

Bi-fluorescence imaging for estimating accurately the nuclear condition of *Rhizoctonia* spp.

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Abstract

Aims: To simplify the determination of the nuclear condition of the pathogenic *Rhizoctonia*, which currently needs to be performed either using two fluorescent dyes, thus more costly and time-consuming, or using only one fluorescent dye, thus less accurate.
Methods and Results: A red primary fluorescence (autofluorescence) of the hyphal cell walls and septa of *Rhizoctonia* spp. with green excitation is evidenced in *Rhizoctonia* spp. This property is exploited and combined for the first time with a conventional DAPI fluorescence to accurately determine the nuclear condition of *Rhizoctonia*. This bi-fluorescence imaging strategy depicted the nuclear condition in *Rhizoctonia* spp. more accurately than the conventional DAPI fluorescence used alone and was validated against isolates previously genotyped by DNA sequencing.
Conclusions: We demonstrated that the bi-fluorescence imaging strategy was safe, accurate and simple to perform and interpret.
Significance and Impact of the Study: The developed bi-fluorescence imaging strategy provides a sensitive tool for determining the nuclear condition of *Rhizoctonia* strains. Its simplicity is a key advantage when there are numerous cultures to be examined.

The form genus *Rhizoctonia* D.C. is regarded as a heterogeneous assemblage of filamentous fungal taxa that do not produce asexual spores and share a number of common features in their anamorphic states (Garcia *et al.* 2006). It includes several of the most devastating crop pathogens such as *Rhizoctonia solani* Kühn [teleomorph = *Thanatephorus cucumeris* (A.B. Frank) Donk]. In the absence of a perfect state, the number of nuclei in the vegetative hyphal cells is one of the anamorphic features that separate the former from other *Rhizoctonia*-like fungi. Anamorphs of *R. solani* are typically multinucleate, whilst *Rhizoctonia* spp., with a *Ceratobasidium* perfect state, are binucleate or more rarely uninucleate (Garcia *et al.* 2006). However, even within the same isolate, the nuclei number can vary from one cell to another in the same hypha (Sanford and Skoropad 1955; Flentje *et al.* 1970). Nowadays, the determination of the nuclear condition in *Rhizoctonia* is a straightforward procedure: applying a nuclear stain that fluoresces upon excitation by UV light, such as DAPI (4′,6′-diamidino-2-phenylindole) and Hoechst Dye 33258, both specific to AT-rich regions, which is visualized with epifluorescence microscopy (Hua'an *et al.* 1984; Kangatharalingam and Ferguson 1991). Accurate determination of hyphal walls and of the septa from a flat thin layer of disentangled mycelium is also necessary, but can be tedious when dealing with a large number of isolates. A solution is to add a fluorophore that binds the hyphal walls. The two most commonly used are Calcofluor White M2R or Solophenyl Flavine 7GFE 500 (Hoch *et al.* 2005). Calcofluor, in

contrast to Solophenyl Flavine, exhibits rapid photobleaching. Moreover, as the respective spectra of Calcofluor and DAPI overlap, the fluorescence signal of Calcofluor lacks contrast. Both dyes are derivatives of the toxic stilbene, although Calcofluor is only classified as an eye irritant (R36), a sensitizing chemical (R42) in reference to the Globally Harmonized System of Classification and Labelling of Chemicals. Commercially available formulations can include counterstains such as Evans Blue or a naphthalene derivative, all substances classified as R45 (carcinogenic). For environmental safety of laboratory workers, one should consider their use only if no alternative method exists.

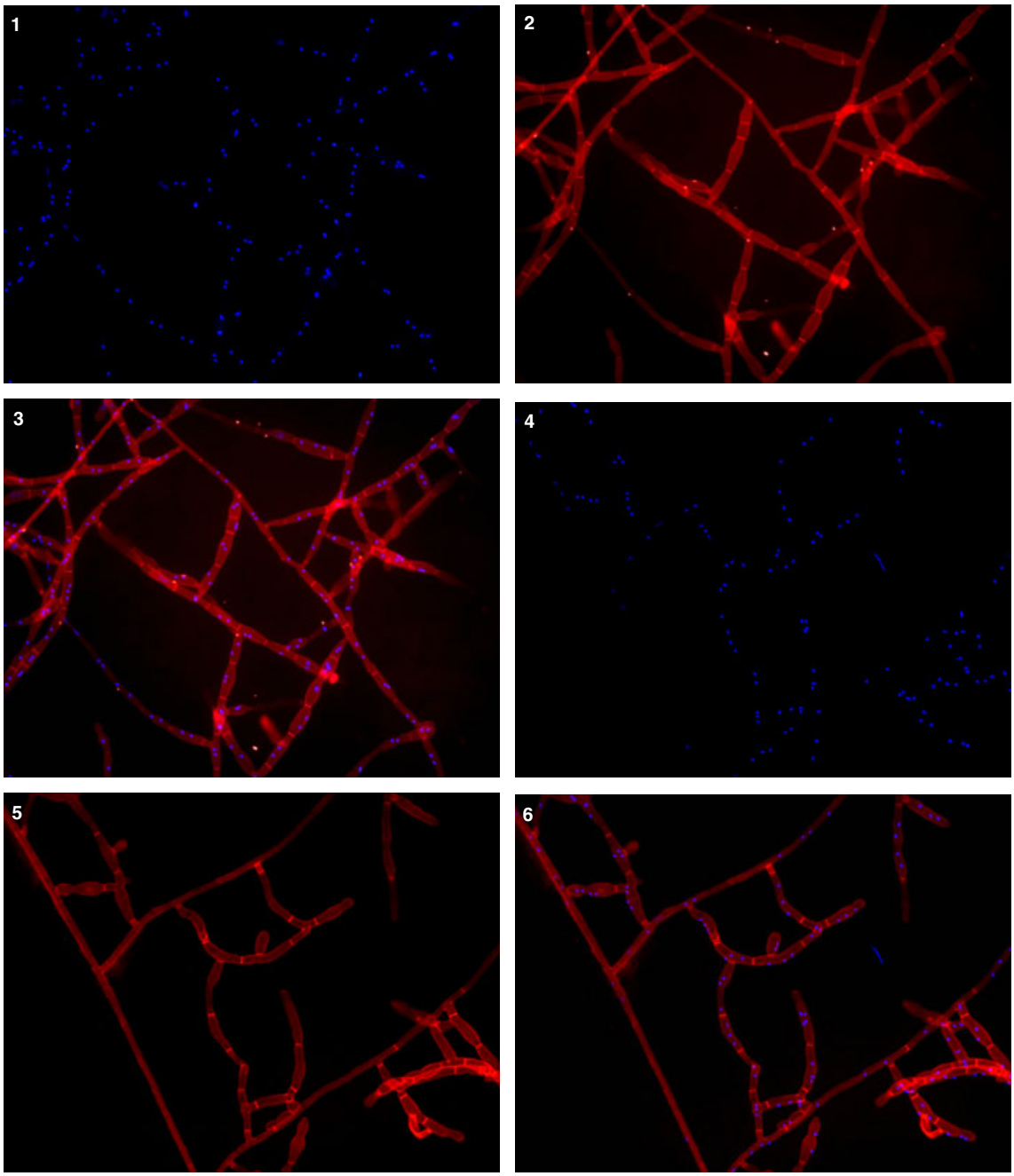
This study describes an alternative method that meets the need to simultaneously and accurately visualize hyphal cells and their respective nuclear number. A red primary fluorescence (autofluorescence) of the hyphal cell walls and septa of *Rhizoctonia* spp. with green excitation was observed during the course of DAPI nuclei observation of *Rhizoctonia* isolates. To our knowledge, red autofluorescence with green excitation has only been reported in hyphae of *Piptoporus betulinus* (Zizka *et al.* 2010). Use of autofluorescence in fungal propagules (Wu and Warren 1984) has not gained much ground in applied microbiology except for fungal detection within plant tissues (Dreyer *et al.* 2006; Zizka and Gabriel 2006). In *R. solani* culture, Wu and Warren (1984) observed autofluorescence in the entire mycelium, but at violet-blue excitation. Red autofluorescence is exploited here in combination with a conventional DAPI fluorescence. The bi-fluorescence imaging proposed depicts safely and cheaply the nuclei number per hyphal cell in *Rhizoctonia* spp. and more accurately than the conventional DAPI fluorescence used alone.

The isolates E06f-11, E06f-12 and E07f-12, identified as *Rhizoctonia* sp. (Caesar *et al.* 2010), were provided by Anthony Caesar at the USDA-ARS-NPRL in the USA. The isolate MIAE00240 was obtained from Microorganisms of interest for Agriculture and Environment (MIAE) Collection (INRA, Dijon, in France) http://www2.dijon.inra.fr/umrmse/ and was previously identified as *Rhizoctonia solani* by Fiers *et al.* (2011) (Table 1).

A small block (0·5 cm^2) with hyphal tips was cut from of a 7-day-old culture grown on potato dextrose agar (PDA) at 28°C and used as an inoculum for the mycelium slide culture according to the method reported by Harris (1986) with the following modification. The agar block was transferred to the centre of a microscope slide that was placed on the top of a U-shaped glass rod. Petri dishes were then incubated in darkness at 28°C for 2 weeks. The microscope slide colonized by the mycelium was incubated in an oven (Merck-Prolabo, Fontenay sous Bois, France) at 37°C for 30 min to remove excess moisture. As agar might provide baseline fluorescence, best observations were made on the mycelium without agar. Then 20 μl of the 1·5 μg ml^{-1} DAPI solution (Vectashield H1200; Vector Laboratories Burlingame, CA) was added to the slide. DAPI hybridization lasted for 15 min. Slides were observed using a Leica DMRAX2 epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Fluorescence images of DAPI-stained nuclei were acquired using the filter A4 for UV light excitation (excitation BP: 340–380 nm; emission BP: 450–490 nm). Autofluorescence was observed under epifluorescence settings with the Texas red filter for green light excitation (excitation BP: 540–580 nm; emission BP: 607–682 nm). The red and blue fluorescence images were acquired separately with a cooled high-resolution CCD camera Hamamatsu Orca AG (Hamamatsu Photonics K.K., Hamamatsu City, Japan) interfaced to a PC running the program Volocity version 5.5 (Perkin Elmer, Courtaboeuf, France). Picture acquisition was under autocontrast with exposure times ranging from 2·5 to 4 s. The total number of nuclei per hyphal cell was estimated with double image overlays using the image analysis software J ver. 1.43, a public domain image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). As a control for

Table 1 Nuclei condition in the four *Rhizoctonia* isolates using the bi-fluorescence imaging strategy in comparison with DAPI method and GenBank accession numbers of their ITS sequences

Isolate/ Anastomosis grouping	ITS GenBank accession number	Range comparison of nuclei number/hyphal cell [min.-max.] based on 50 hyphal cells as counted using bi-fluorescence		Number of nuclei/hyphal cell (mean ± SE), and range [min.-max.] using bi-fluorescence *n* = number of hyphal cells counted
		DAPI	Bi-fluorescence	
E06F11/nd	HQ738653 (this study)	[1–4]	[1–3]	2·0 ± 0·4 [1–3] *n* = 284
E06F12/AG4	JF831083 (this study)	[1–4]	[1–5]	2·9 ± 0·9 [1–8] *n* = 253
E07F12/nd	HQ738654 (this study)	[1–4]	[1–3]	2·0 ± 0·2 [1–3] *n* = 144
MIAE00240/AG3	HQ898715 (Fiers *et al.* 2011)	[1–6]	[1–12]	5·6 ± 1·9 [1–12] *n* = 136



Figures 1–6 Epifluorescence microscopy of the binucleate *Rhizoctonia* E06f11 (1–3) and the multinucleate *Rhizoctonia solani* E06f12 (4–6). DAPI images (1 and 4); autofluorescence images (2 and 5); overlapped bi-fluorescence acquisition images (3 and 6). Magnification × 200.

autofluorescence, unstained mount preparations were mounted either with antifade media (H1000; Vectashield, Vector Lab) or buffered glycerol (M1289; Sigma Aldrich, Saint Quentin Fallavie, France) and observed using the same protocol. Amplification and direct sequencing of the two internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S of each isolate were carried out as described in Fiers *et al.* 2011.

Nuclei in all *Rhizoctonia* isolates were brightly stained by DAPI under UV excitation (Figs 1 and 4), whilst hyphal walls and the septa were barely visible within overlapping hyphae. Therefore, hyphal cell counts were approximate, as was the range of nuclei number per hyphal cell (Table 1). The same hyphal walls and septa exposed to green excitation fluoresced intensely red with no photobleaching (Figs 2 and 5). This phenomenon was

to be endogenous as it was observed in control preparations mounted with antifade media or buffered glycerol. By using bi-fluorescence imaging, the exact number of nuclei per hyphal cell as well as its range could be assessed for each isolate (Figs 3 and 6, Table 1). Bi-fluorescence results confirmed the nonuniform distribution of the nuclei within hyphae at the isolate level as reported by Sanford and Skoropad (1955) (Table 1). Multinucleate condition in the isolate E06f-12 confirmed it to be *R. solani*, and this identification was subsequently corroborated by the highest BLAST similarity score (100%) of its ITS sequences to other accessions of *R. solani* in GenBank (Table 1). The isolates E06f-11 and E07f-12 were binucleate, and thus, *Rhizoctonia* spp. and their ITS sequences had the highest BLAST similarity scores (94%) with binucleate isolates in GenBank (Table 1). The exact nature of the autofluorescence we describe needs further investigation. The potential sources of cell autofluorescence in fungi could be chitin, glucans, flavines, lipofuscins, porphyrin, lignin and other substances (Billington and Knight 2001). As it persisted after alkali treatment using 10% KOH, chitin, β 1-3 glucans, among others might be good candidates (Fontaine *et al.* 2000), although the excitation spectrum of pure chitin did not correspond exactly to the one used for autofluorescence here (Jabaji-Hare *et al.* 1984).

The advantages of this bi-fluorescence imaging strategy over current double staining methods include simplicity and reduced cost, laboratory worker exposure and environmental hazard. This method depicted the nuclear condition in *Rhizoctonia* spp. more accurately than the conventional DAPI fluorescence used alone. A direct application of red autofluorescence would be the visualization of hyphal fusion during anastomosis—an essential means of classifying isolates of *Rhizoctonia solani*. Bi-fluorescence imaging may prove to be of great practical significance for research in various fields of microbiology.

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